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TITLE: The Role of Perfluorocarbons in Mitigating Traumatic Brain Injury

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14. ABSTRACT

Neurological injury [brain and cord] is always accompanied by tissue deprivation of glucose and oxygen (ischemia/hypoxia). Most of the damage seems to be mediated by mechanisms that follow the initial injury (secondary mechanisms). Perfluorocarbons (PFCs) are one of the methods by which oxygen delivery to tissue can be achieved after injury. The rationale for PFCs in traumatic brain injury has been well established in animal studies and early phase 2 clinical trials. Currently three perfluorocarbons are available in the United States for testing, but none of these have been FDA approved and only for one of them – Oxycyte has the process of application for FDA approval even been commenced. For the third generation perfluorocarbon (Oxycyte) a possible side effect that has emerged in humans is transient mild thrombocytopenia. It is uncertain at this time whether this side effect will prove to be a limiting factor, which may jeopardize the use of these compounds as a class, or just affect Oxycyte in particular following traumatic brain and spinal cord injury. Any agent that might exacerbate thrombocytopenia in intracranial hemorrhage into traumatic contusions is dangerous for obvious reasons. The purpose of this grant therefore is to cross compare the safety and efficacy of three perfluorocarbons namely Oxycyte, Perftec and Oxygent. We assessed these 3 PFC agents in two head injury models (1) new PENETRATING brain injury animal model (human gunshot wound to head) and (2) closed severe rat TBI –Fluid Percussion Injury (human car crash) with a secondary Hypoxic insult. We measured how the PFCs alter the ability of the injured brain (1) to use glucose, oxygen and (2) lower cell death caused by injury, (3) effect on blood clotting. First, we did not find any evidence of impairment of blood clotting in rats with TBI after treatment with PFCs unlike in humans. Secondly, the PFCs modestly improved use of oxygen, surprisingly even glucose; however these improvements did not translate into fewer dead cells. Using novel techniques, we found that there is persistent reduction in blood flow to brain after injury. For the first time we also showed by electron microscopy that PFCs appear to improve membrane integrity. Although we could not find them beneficial in this model, PFCs can be improved.

15. SUBJECT TERMS: Penetrating ballistic brain injury, ischemia, hypoxia, perfluorocarbon, cell death, perfusion.

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(2) SECTION I

- A brief introduction covering the purpose and scope of the research effort.

INTRODUCTION AND GRANT RATIONALE

Perfluorocarbons are one of the methods by which oxygen delivery to tissue can be achieved after injury. Neurological injury [brain and cord] is always accompanied by tissue ischemia/hypoxia and much of the damage seems to be mediated by this secondary mechanism. The rationale for perfluorocarbons in traumatic brain injury has been well established in animal studies and early phase 2 clinical trials. Currently three perfluorocarbons are available in the United States for testing, but none of these have been FDA approved and only for one of them – Oxycyte has the process of application for FDA approval even been commenced. For the third generation perfluorocarbon (Oxycyte) a possible side effect that has emerged in humans is transient mild thrombocytopenia. It is uncertain at this time whether this side effect will prove to be a limiting factor, which may jeopardize the use of these compounds as a class, or just affect Oxycyte in particular following traumatic brain and spinal cord injury. Any agent that might exacerbate thrombocytopenia in intracranial hemorrhage into traumatic contusions is dangerous for obvious reasons. The purpose of this grant therefore is to cross compare the safety and efficacy of three perfluorocarbons namely Oxycyte, Perftec and Oxygent. We assessed these 3 PFC agents in a new **PENETRATING brain injury animal model, devised at WRAIR (the Tortella PTBI model**) and in **closed severe rat TBI –Fluid Percussion Injury…FPI—**with and without a secondary Hypoxic insult, for the first time, with such agents.

The 4 specific aims are stated below:

Aim 1: PFC will be effective in mitigating penetrating TBI, as tested in the WRAIR/Tortella model of penetrating ballistic-like brain injury (PBBI), with acute brain histology, at 24 -72 hours after injury, in the rat.

Aim 2:

A. Two doses of PFC, given 24 hours apart will be safe and effective in mitigating secondary ischemic damage, superimposed upon severe closed TBI in the rat.
B. TEG will be performed in collaboration with the Wallace Coulter Platelet Function laboratory at the University of Miami, in both FPI rat models, and in Human blood In Vitro.

Aim 3. PFC's will improve both

A. Oxygen consumption ($CMRO_2$) and
B. Glucose use, in the rat brain, after PTBI**.**

Aim 4. PFC will improve cell survival, in an in vitro model of mild TBI, when applied in the supernatant culture medium.

Our letter of award was made in August 2011 and in this ***report,*** we outline progress that has been made in the **2.5-year period (Sept 2011-March 2014)**

(3) Section II - A brief description of overall progress to date plus a separate description for each task or other logical segment of work on which effort was expended during the report period. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. If this award includes the recruitment of human

subjects for clinical research or a clinical trial, report progress on subject recruitment (i.e., number of subjects enrolled versus total number proposed).

The **SCHEDULE OF WORK** from the grant application is attached below, and the status of each task is reported in the following tables.

SCHEDULE OF WORK----PROJECT TASKS

TITLE: The Role of Perfluorocarbons in Mitigating Traumatic Brain Injury.
PI: M. Ross Bullock, MD, PhD, University of Miami Miller School of Medicine, Department of Neurological Surgery, 1095 NW 14th Terrace LPLC 3-18, Miami, Florida 33136

1. TASK 1: Initial Preparation/Logistics (Months 1-2)

   a. **Hire and assemble a research team, purchase equipment, and reagents; prepare the logistics for experiments over the following 2 years**
   b. **With guidance from USAMRMC ACURO we will write, review protocols for animal studies and obtain approval by both DOD and the University of Miami Animal care and use committee.**
   c. **Order reagents, surgical supplies, hire a post-doctoral fellow, technologist, and train staff 1-3 months**
   d. **Ordering of the Animals, as needed throughout the 2-year project, see Grant chart, below.**
   e. **Obtain the PBBI instrument, with help from Tortella lab postdoc, Dr Leung conduct PBBI.**

2. **TASK 2: Aim 1.** ***PFC will be effective in mitigating Penetrating TBI, as tested in the WRAIR/Tortella model, with acute brain histology, at 24 and 72 hours after injury, in the rat.*** **(Months 2-10).**

   a. **Start the experiment with reproducible PBBI and the establish treatments of PFCs**
   b. **We will begin the histopathological and immunocytochemical staining and analyses during this time frame and should be completed with the majority of the analysis completed for specific Aim 1 by month 12,** Task 2 50 male Sprague Dawley rats

3. **TASK 3: Aim 2.** ***Two doses of PFC, given 24 hours apart will be safe and effective in mitigating secondary ischemic damage, superimposed upon severe closed TBI in the rat.*** **(Months 10-16)**

   a. **Model is fluid percussion injury (FPI TBI) +hypoxia treatment with different PFCs.**
   b. **The 'run in' group will be 2-3 animals per task to address any technical difficulties.**

   a. –Training of personnel. Month 6, Animal surgeries…months 7-9, **Task 3 60 male Sprague Dawley rats**
   b. **Histopathology….months 8—14, Data analysis and final report –months 12—16.**

4. **TASK 4: Aim 3.** ***PFC's will improve both oxygen consumption ($CMRO_2$) and glucose use, in the rat brain, after TBI.***

   a. **Model is FPI TBI with PFC to assess oxygen consumption ($CMRO_2$) and 2-DG uptake.**
   b. **–Training of personnel. Month 10. Animal surgeries…months 10—17** Task 4 160 male Sprague Dawley rats

c. **Histopathology….months 14—20, Data analysis and final report –months 19-22.**

5. **TASK 5: PFC will improve cell survival, in an *in vitro* model of MILD TBI, when applied in the supernatant culture medium.**

   a. ***In vitro* experiment to explore if PFC mediated neuroprotection is via membrane stabilizing effect.—month 10,** Task 5 20 female time pregnant Sprague Dawley rats.
   b. **experiments…months 11—12, data analysis and reports…months 13—15**

6. **TASK 6: Aim 5: *PFC mitigating TBI induced cognitive deficits, as tested by Morris Water maze***

   a. **Identify the most effective PFC in previous Aims, and compare with Oxycyte in a FPI TBI model with cognitive component**
   b. **experiments…months 22—24, data analysis and reports…months 23—24** Task 6 30 male Sprague Dawley rats.

7. **TASK 7; Interim Analysis**

   a. **Interim statistical analysis of the data obtained from different aims of the study**
   b. **Quarterly progress reports (every 3 months) and annual reports to be written for DOD reviewers.**

8. **FINAL DELIVERABLES**

   a. **Final report to DOD CDMRP and initial manuscripts as available,**
   b. **Detailed manual of operations for surgery, behavior and histopathology**
   c. **Manuscripts to journals, detailing results of each specific aim.**

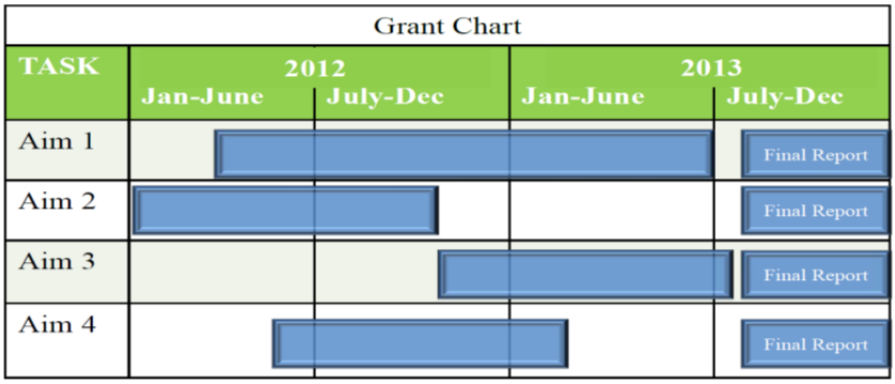


Table 1 Animal use Gantt chart

We have completed all experiments involving animal use, according to the Schedule of Work (SOW) …

1) Several abstracts and manuscripts, arising from these studies are in preparation (see appendix). The summary of all work is shown as Fig.1 and corresponds to the image quadrant of the Quad slide.

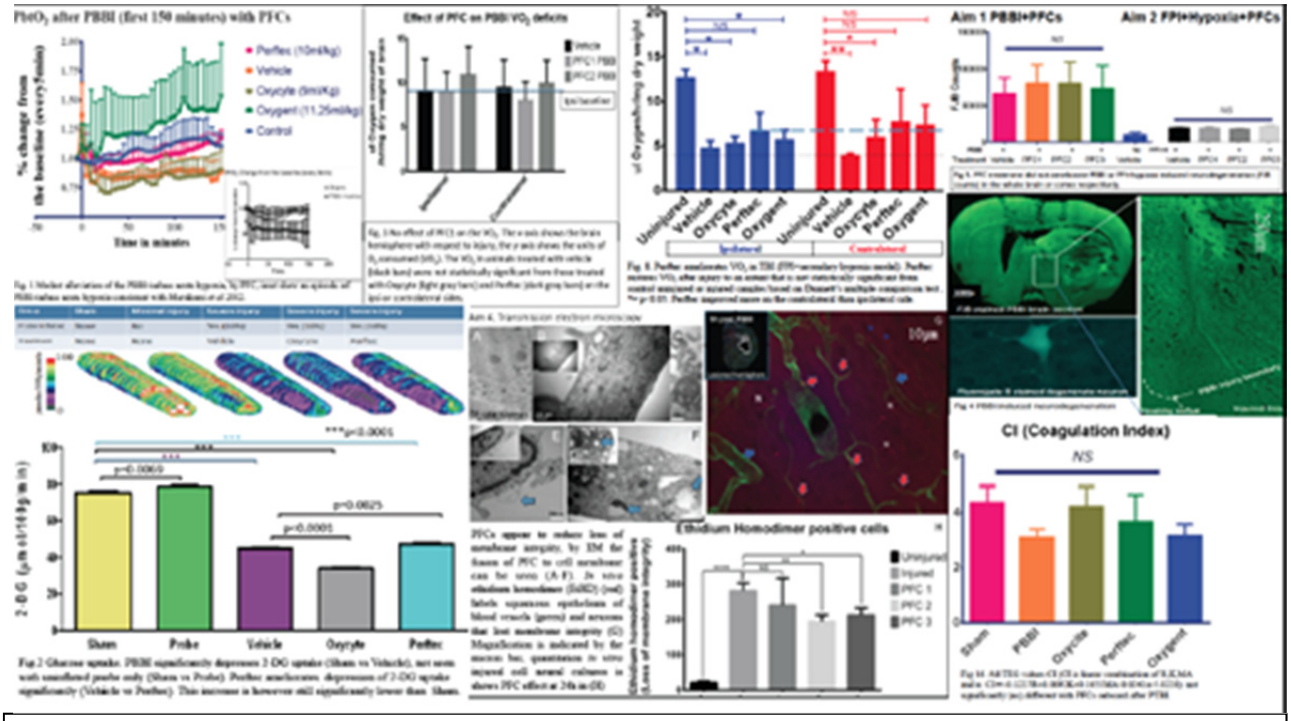
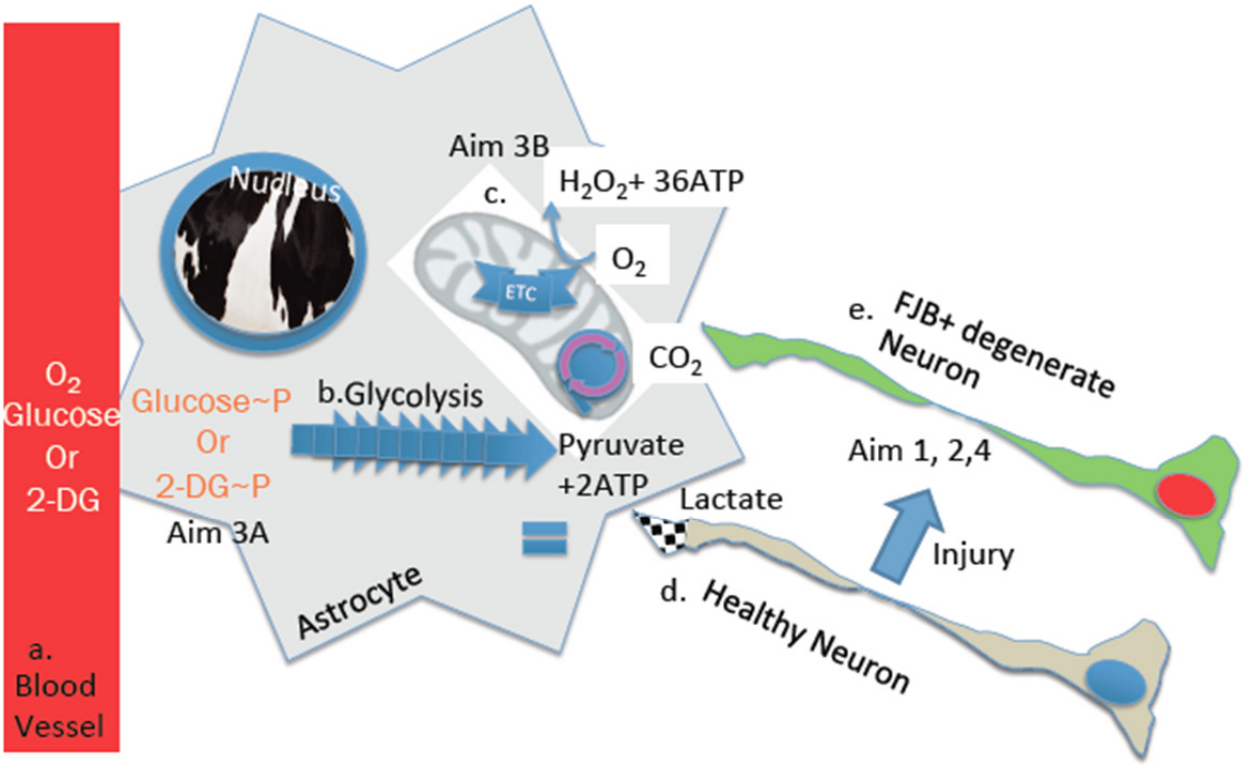


Fig.1. A composite of all figures show that PFCs improve pBtiO2 (1), glucose uptake (2), no improvement of VO_2 after PTBI, but modest improvements in VO_2 (3), some membrane preservation (4) but these effects do not translate to mitigation of neurodegeneration (5) and did not alter trauma induced coagulopathy (6) in rat.

Figure 2 -The processes interrogated by the Aims of this proposal are shown in this schematic. Glucose and oxygen are transported into the brain by vasculature. PFC facilitates this. Glucose is taken up by the cells (Aim 3A) and oxidized via consumption of oxygen (Aim 3B); these metabolic processes keep cells alive. Following injury interruption of these processes could lead to neurodegeneration (Aims 1 and 2).



Aims: The simplified schematic shows the aims of this proposal interrogating processes underlying neuronal survival. A blood vessel (a, left) supplies oxygen and glucose (or 2-deoxy glucose) to the brain, glucose is phosphorylated and broken down by glycolysis (b, middle) in cytoplasm to generate pyruvate/lactate. The pyruvate enters mitochondria undergoes tricarboxylic acid and oxidative phosphorylation resulting in consumption of oxygen (Aim 3B) and ATP generation (c). Alternatively lactate is released and is used by a healthy neuron (d). Disruption of these processes following injury results in neurodegeneration Aim 1,4 (e, right).

OVERALL EXECUTIVE SUMMARY.

No beneficial neuroprotective effect, of any of the 3 PFC tested, was seen, as judged by FJB positive cell counts.

Fig 3A (FJB counts in PBB+PFC), Fig 3B (FJB Counts in FPI+hypoxia), PBBI+PFC image of FJB in Fig 4-9 and lectin labeling of PBBI brain.

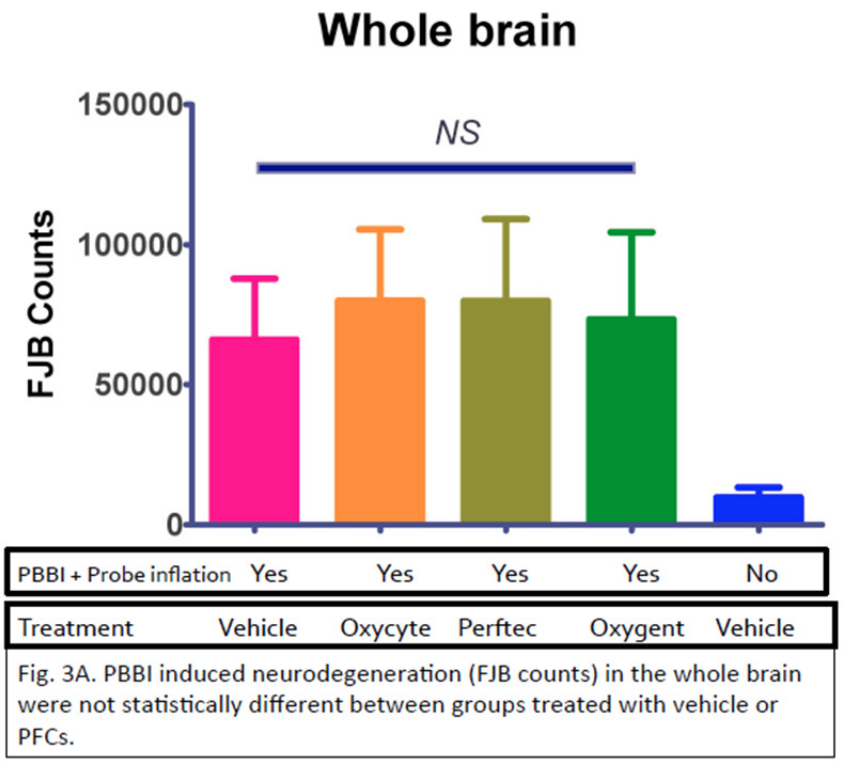


Fig. 3A. PBBI induced neurodegeneration (FJB counts) in the whole brain were not statistically different between groups treated with vehicle or PFCs.

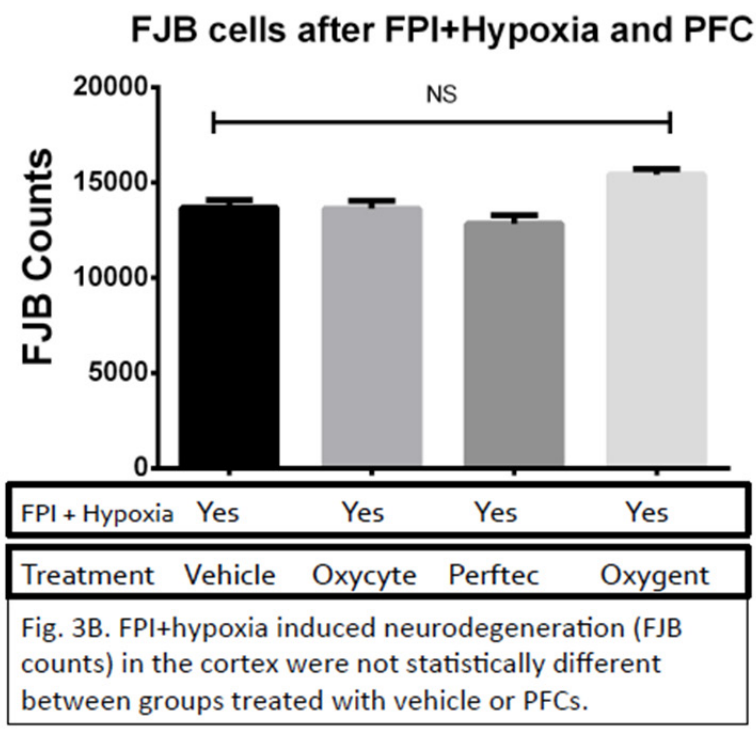


Fig. 3B. FPI+hypoxia induced neurodegeneration (FJB counts) in the cortex were not statistically different between groups treated with vehicle or PFCs.

Figure 4 - FJB stained brain section to illustrate fluorescent green-stained degenerate neurons right panel), the numbers of which were assessed by unbiased stereology, by a blinded investigator. (high power view, right, lower panel). This data suggest that PBBI-induced neurodegeneration at 24 -72h could not be reduced by use of intravenous PFCs at 30 minutes post injury. 2. These studies have completed the most detailed histopathological analysis done to date of the effect of the PTBI model (i) upon neuronal degeneration, and (ii) upon the VASCULATURE. We have observed a progressive increase in the severity of the degree of vascular impairment, after PTBI, over the first week, using the tomato

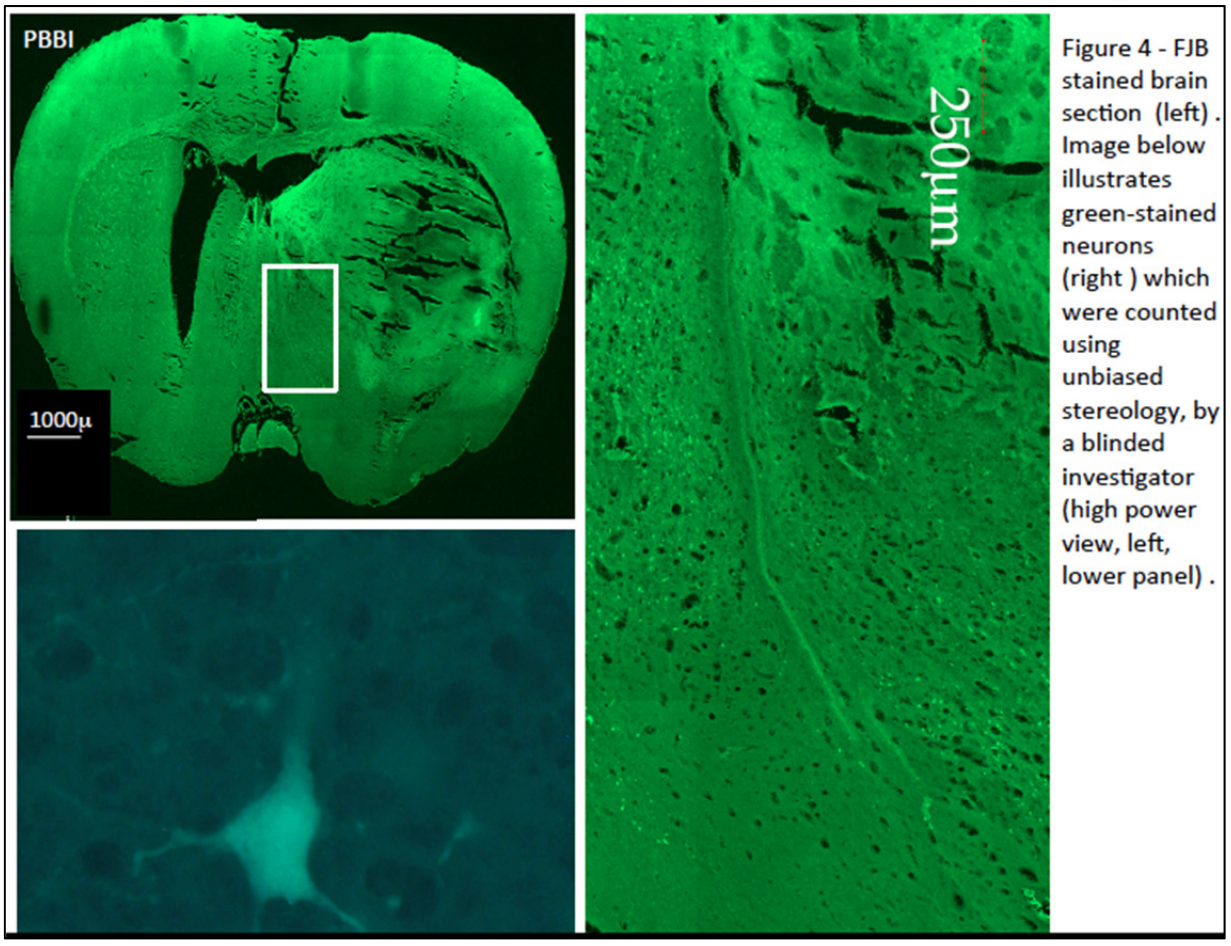


Figure 4 - FJB stained brain section (left) . Image below illustrates green-stained neurons (right) which were counted using unbiased stereology, by a blinded investigator (high power view, left, lower panel) .

red lectin labeling of vasculature in combination with tissue clearing and a newly developed Ultramicroscopy method[1]. We have not yet demonstrated, but loss membrane integrity of cells lining vasculature, vasospasm could be possible explanations for the progressively worsening vascular impairment, seen clearly in Fig 5 below. We speculate that damage to microvascular, occlusions at the capillary and regulatory penetrating arteriolar levels, are responsible for the outcomes (see Fig. 26 also). More studies are needed, possibly with ultrastructure to resolve this mechanism and further qualitative vessel counting studies to make a quantitative analysis. **Our data suggests a central role for progressive microvascular impairment, as a major cause of the neurodegeneration, after PTBI** and could underlie the tissue loss observed by Williams et

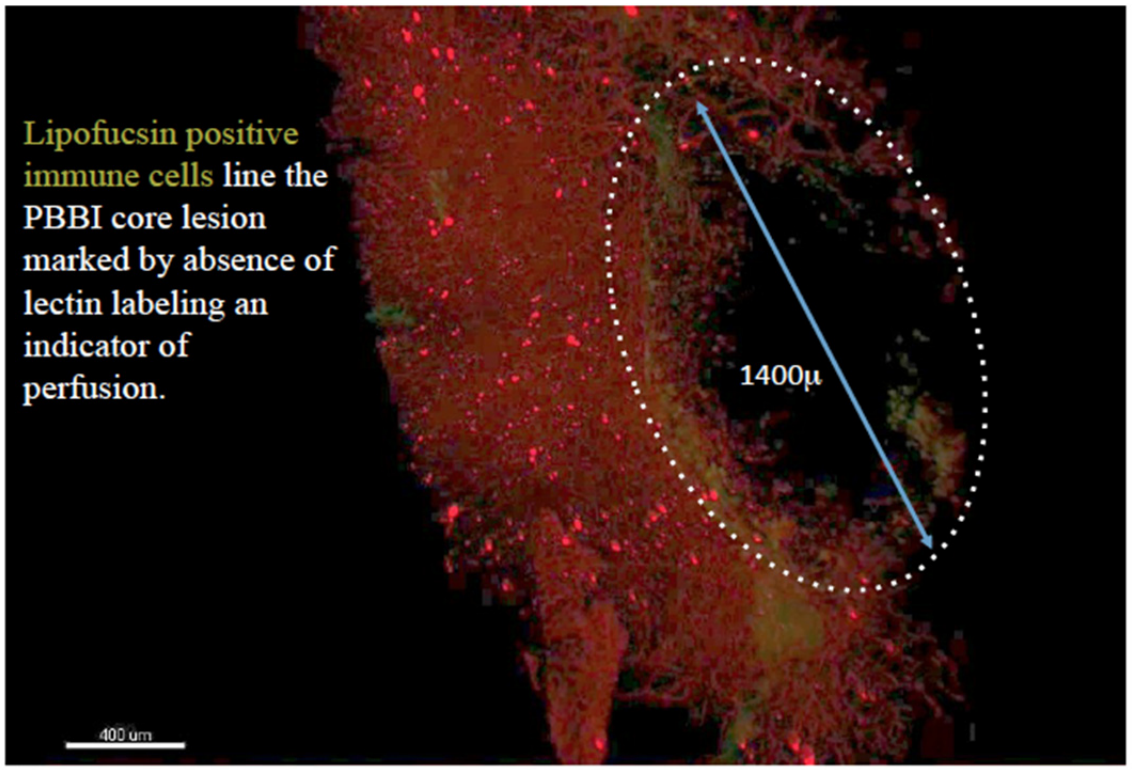


Fig.5 Lectin labeled (red) rat brain after PTBI Even at 1 week post PBBI the core of the PBBI lesion is a ~1.4mm wide ellipsoid of hypo perfused tissue that is lined lipofucsin positive immune cells (yellow autofluorescence).

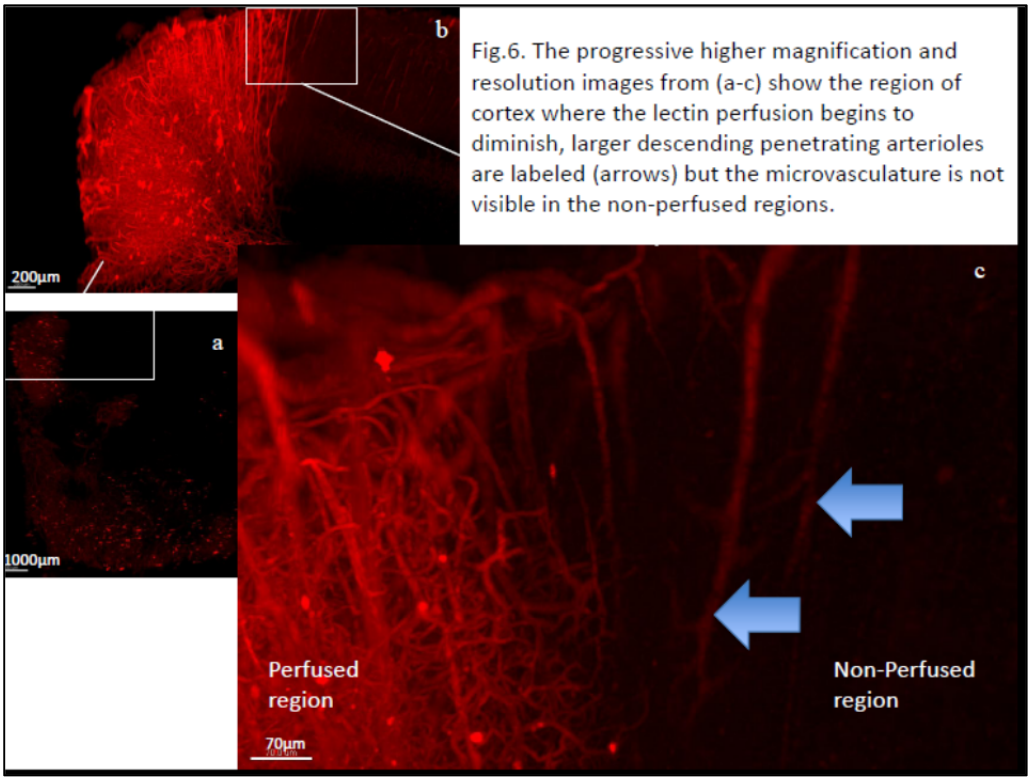


Fig.6. The progressive higher magnification and resolution images from (a-c) show the region of cortex where the lectin perfusion begins to diminish, larger descending penetrating arterioles are labeled (arrows) but the microvasculature is not visible in the non-perfused regions.

al 2005[2]. Figures, 5, 6—lectin vascular labeling.

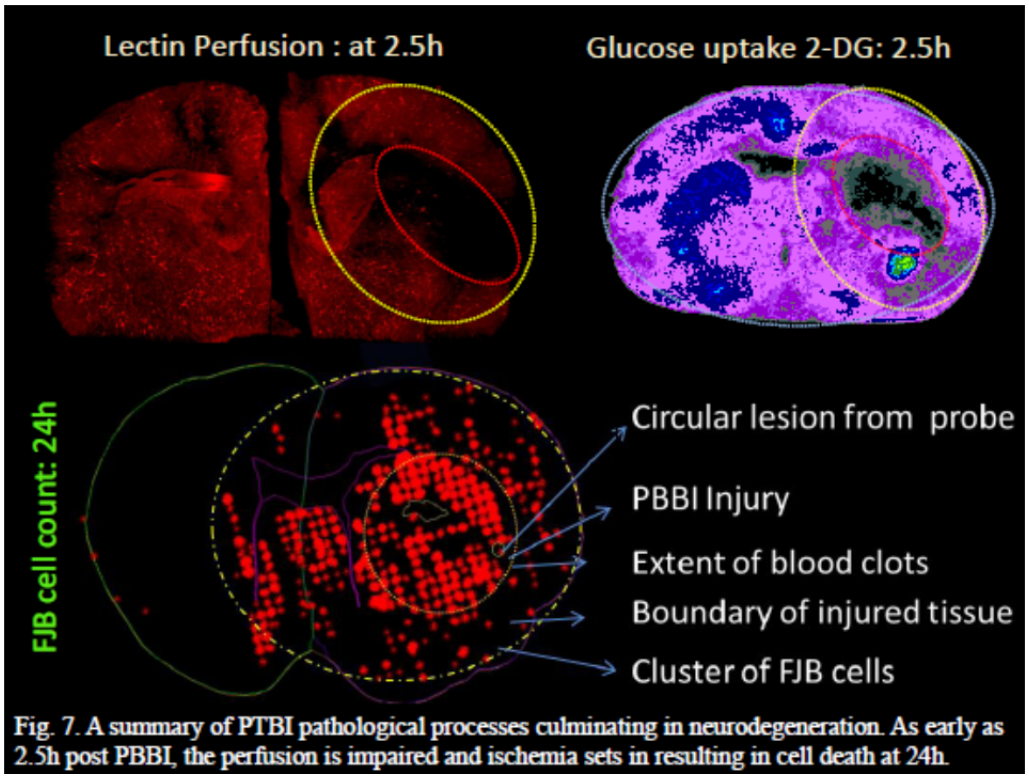


Fig. 7. A summary of PTBI pathological processes culminating in neurodegeneration. As early as 2.5h post PBBI, the perfusion is impaired and ischemia sets in resulting in cell death at 24h.

Ultramicroscopy allows visualization of the entire lesion in an unprecedented quantifiable manner. There is a co-incidence of hypo perfusion, failure of glucose uptake and neurodegeneration in this model. Further analyses will assess the relationship between vascular occlusion, and cell death in this PBBI model.

Progress with Aim 2

The FPI+Hypoxia studies with Oxycyte, Oxygent and Perftec and Vehicle are complete. The treatment with PFCs did not reduce incidence of neurodegeneration. The cell death was 5x less than that seen with PBBI, however unlike with FPI alone the combination with hypoxia proved to be beyond the capacity of PFC to mitigate neurodegeneration (see Fig 3B and 9A-9D). The confocal images on left are higher magnifications of images on right. Yellow arrows indicate the representative FJB cell; the

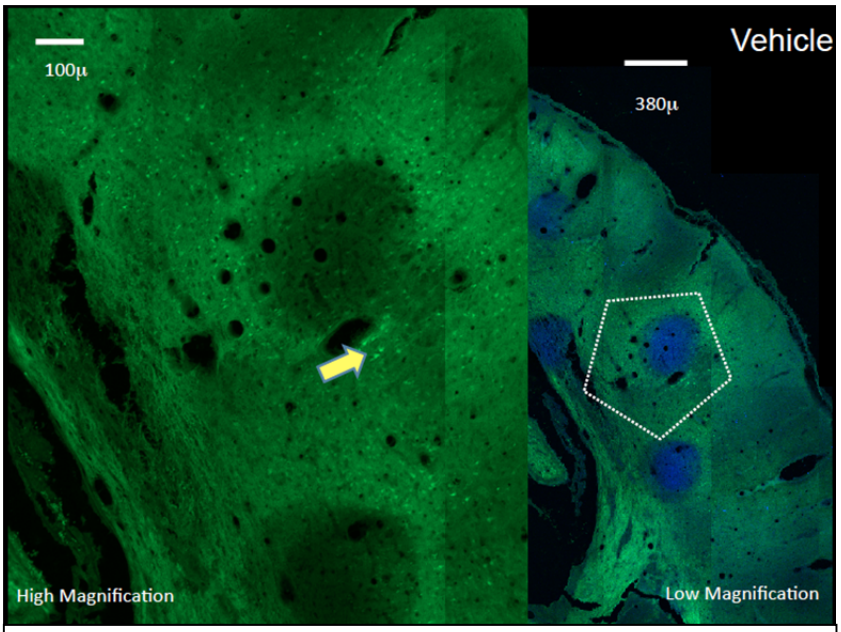
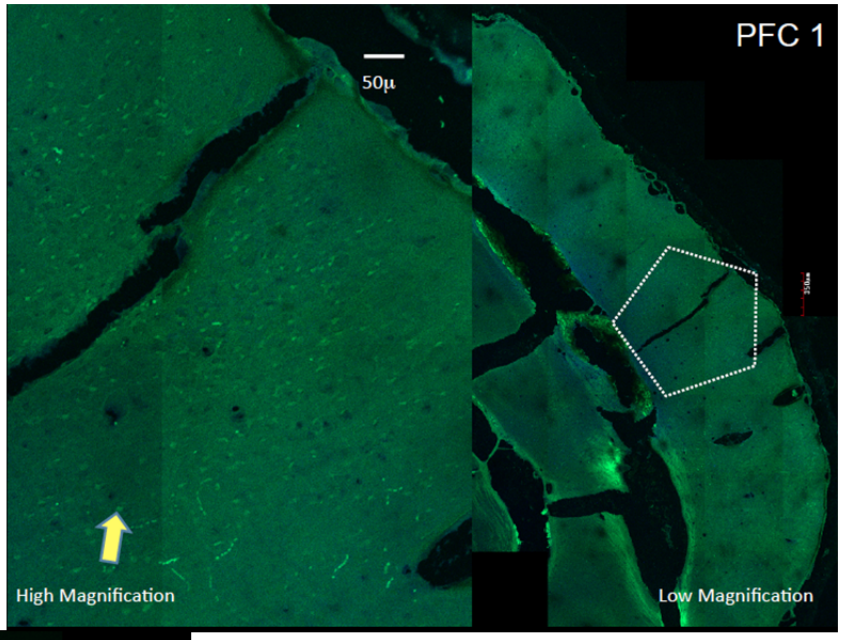


Fig. 8 Vehicle treated animal with FPI+Hypoxia stained with FJB shows degenerated neurons at (left) cortical area (right)

pentagon shows the area of high magnification as well as spread of FPI+Hypoxia induced neurodegeneration. Six slides 60µm apart were analyzed by unbiased stereology and the n=5 per group. The nature of the PFC is indicated on the top right. In all groups the area of FJB cells ranged between 1.5-2.2mm^2 which is twice that seen with FPI alone Das *et al.*, 2011; White *et al.*, 2013). Further this study expose the PFC limit on mitigation of neurodegenration and metabolic stress and is different from PFC mediated metabolic stress relief following FPI.[3-5]



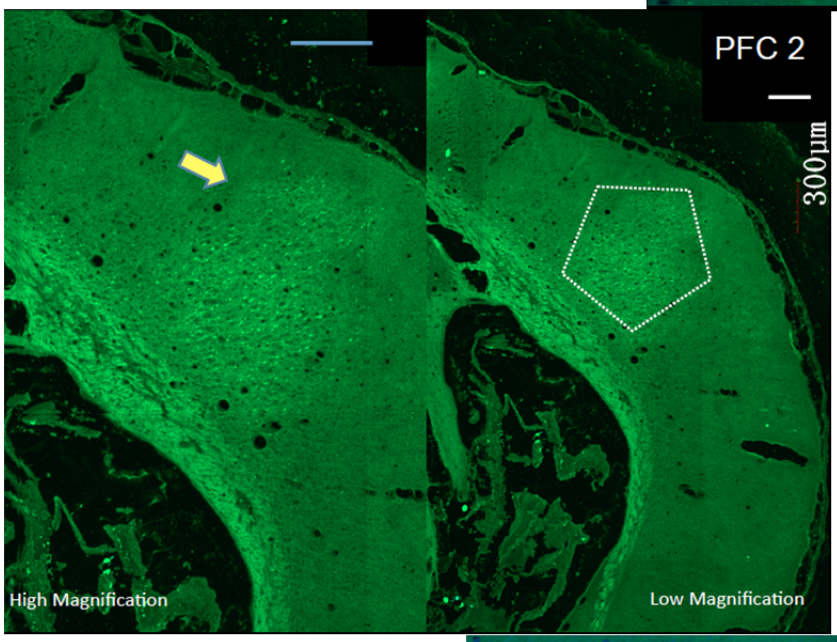
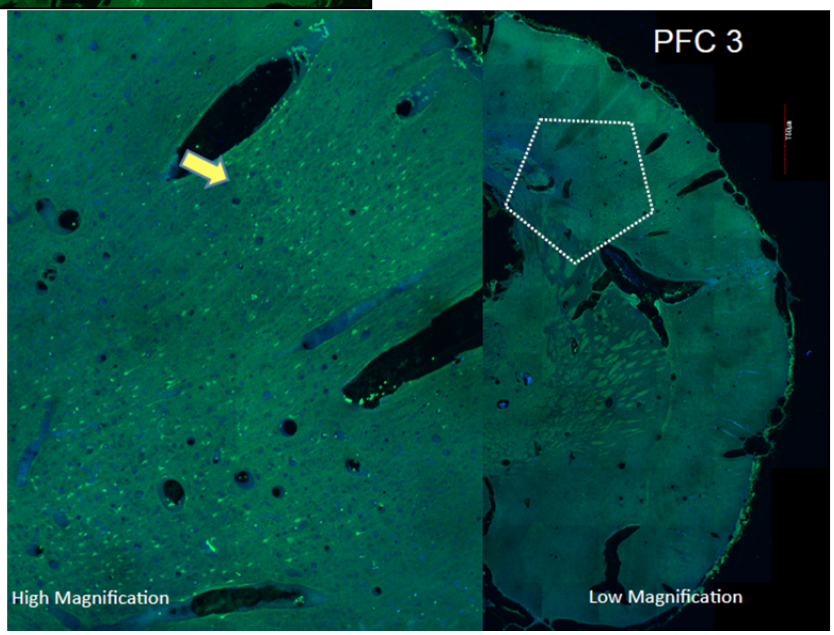
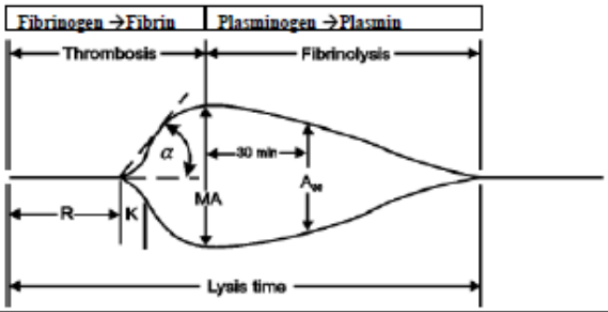


Fig.9. The high magnification images of representative sections with neurodegeneration after FPI+hypoxia and treatment with PFC1 (above), PFC2 (middle) and PFC3 (below) show no differences in cortical area labeled with FJB.



AIM 2B –EFFECT OF PFC UPON PLATELET FUNCTION, AND COAGULATION PARAMETERS.

This part of the Aim 2 is to investigate the status of platelets after exposure to PFCs in rats with TBI. Below, we show selected data from the TEG results from rats (n=6 per group) undergoing (1) TBIs without PFCs (2) only PTBI, with 3 PFC's, given 30 mins after PTBI, and blood was sampled 2.5 hrs, after injury.



Instrumentation	TEG	Clinical meanings
Clot time (period to 2mm amplitude)	R	Initiation phase of enzymatic factor activity
Period from 2 to 20 mm amplitude	K	Potentiation phase of enzymatic factors yielding clot strengthening. This Represent the thrombin cleavage of fibrinogen into fibrin
Alpha angle	α (slope between R and K)	Rate of clot strengthening through polymerization of available fibrinogen. This also represent the thrombin cleavage of fibrinogen into fibrin.
Maximum Amplitude	MA	Functional contribution to clot strength through GP IIb-IIIa receptor interaction with fibrin
Clot strength	G	Overall total clot strength resulting from all coagulation interactions, calculated from amplitude (A), G=(5000×A)/(100×A). The process of clot dissolution or fibrinolysis, leads to a decrease.
Overall coagulation status (Coagulation Index)	CI	CI is linear combination of R,K,MA and α. CI=-0.1227R+0.0092K+0.1655MA-0.0241α-5.0220

Fig 10. The schematic shows the parameters that can be measured using thromboelastography (TEG), the table lists parameters, symbol, clinical interpretation.

The CI—Coagulation index data is the most reliable "overview" of coagulation, and NO significant effect, was seen, for any of the PFC's. (See below, Fig16) this suggests no harmful pro-or anti-coagulant effect, of these compounds, in rats, after a severe brain injury. Further consistent with our previous report that platelets were not aggregated in liver, spleen or lungs, Oxygen Biotherapeutics presented at Military Health System Research Symposium (MHSRS) 2013 Ft. Lauderdale, that radio labeled platelets mature into radiolabeled microparticles and cannot be detected in tissues.

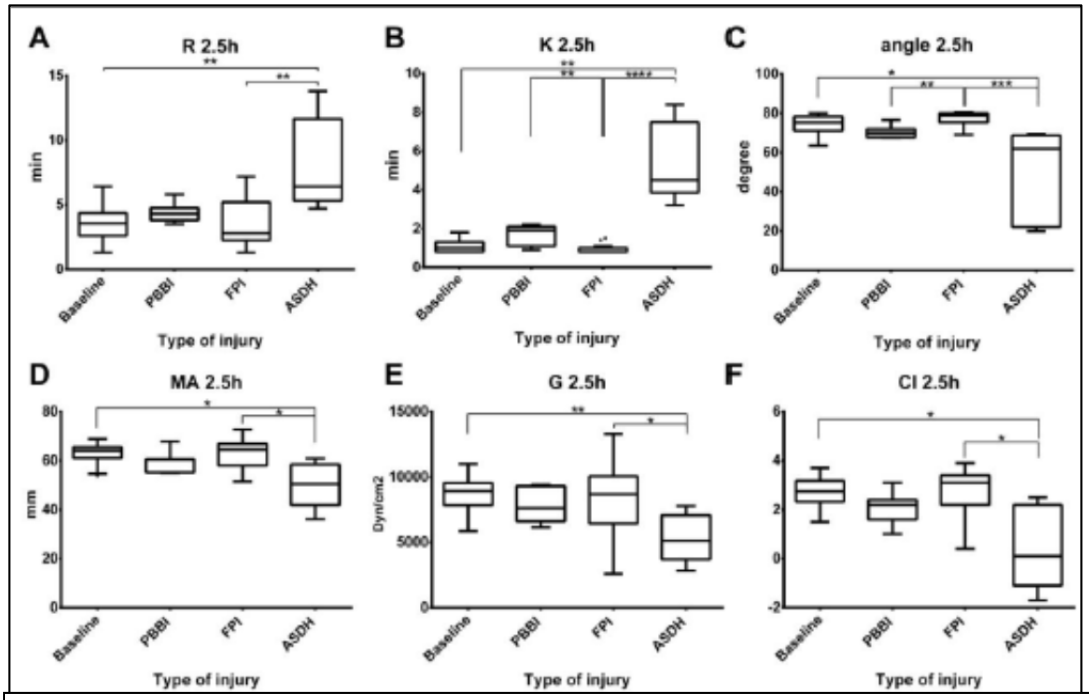


Fig. 11 Difference of TEG parameters (2.5h) among different injury models. More that PBBI and FPI, acute subdural hematoma (ASDH) showed significant impairment in enzymatic coagulation (A), thrombin cleavage of fibrinogen into fibrin (B,C), clot strength (D,E) and overall coagulation status (F), after 2.5h of injury induction. *p<0.05 **p<0.01 ***p<0.001, ****p<0.0001

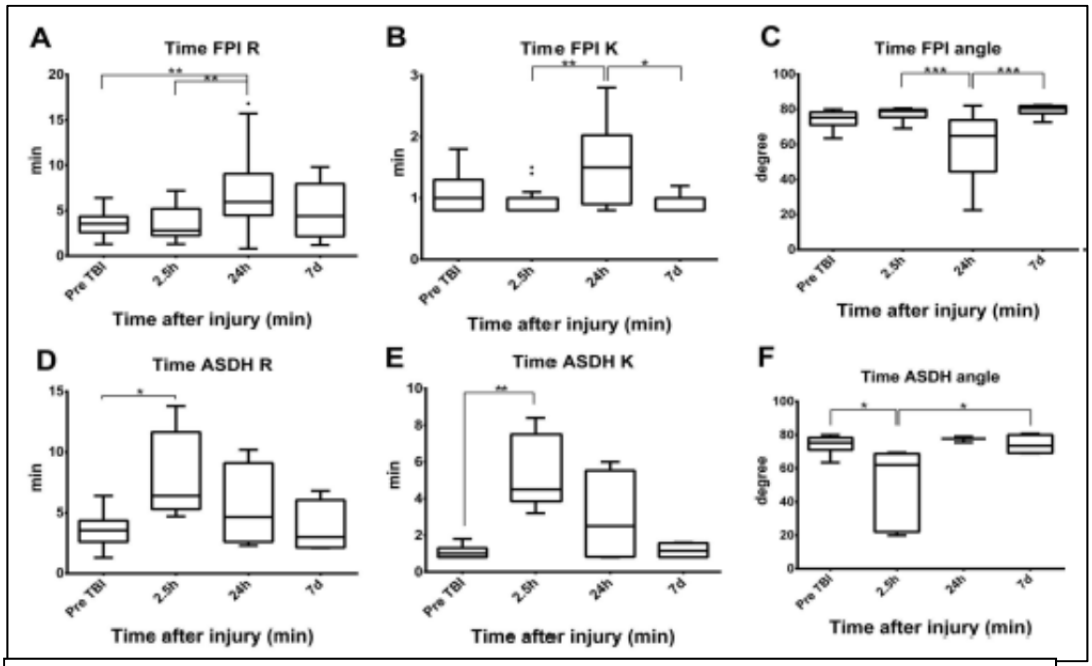


Fig 12. TEG values in different time points in FPI and ASDH rat models. In ASDH, the peaks of dysfunction on enzymatic coagulation (D) and fibrin dysgenesis (E, F) were at 2.5h after injury. Whereas, these peaks in TBI were at 24h after injury (A,B, and C). *p<0.05 **p<0.01 ***p<0.001

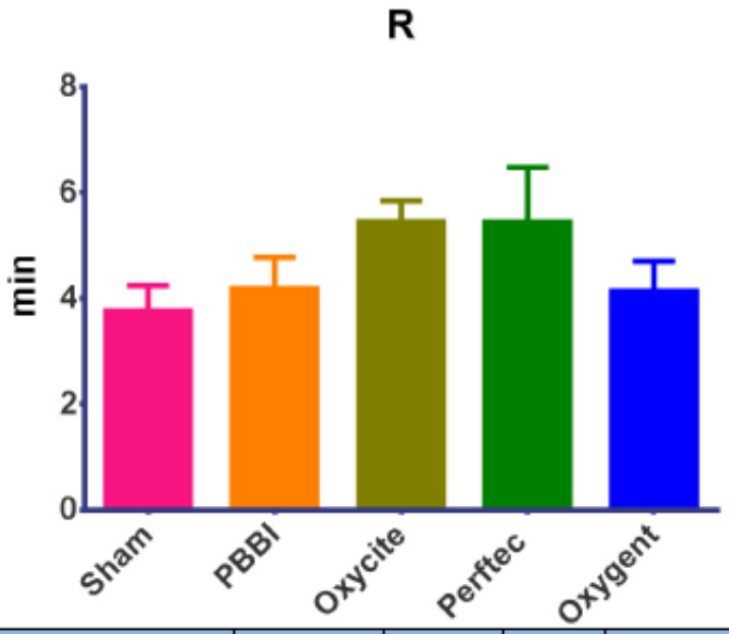


Fig 13. TEG values for R (Initiation phase of enzymatic factor activity) not significantly different with PFCs onboard after PTBI.
using thromboelastography (TEG), the table lists parameters, symbol, clinical interpretation.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
Sham vs. PBBI	-3.714	No	ns	> 0.9999
Sham vs. Oxycite	-11.30	No	ns	0.1020
Sham vs. Perftec	-7.614	No	ns	0.6100
Sham vs. Oxygent	-2.571	No	ns	> 0.9999

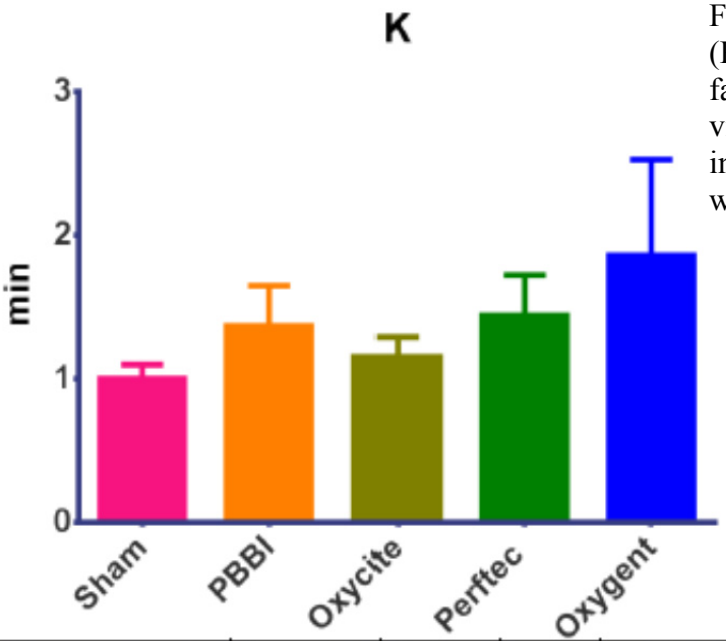
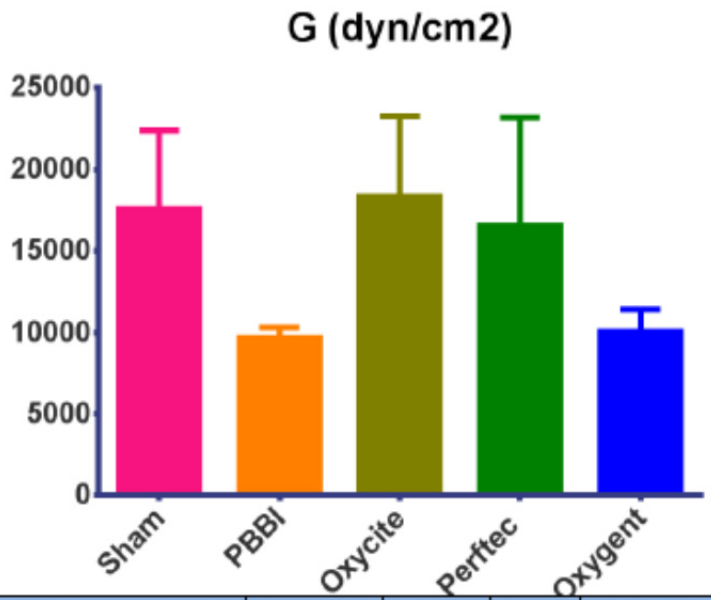


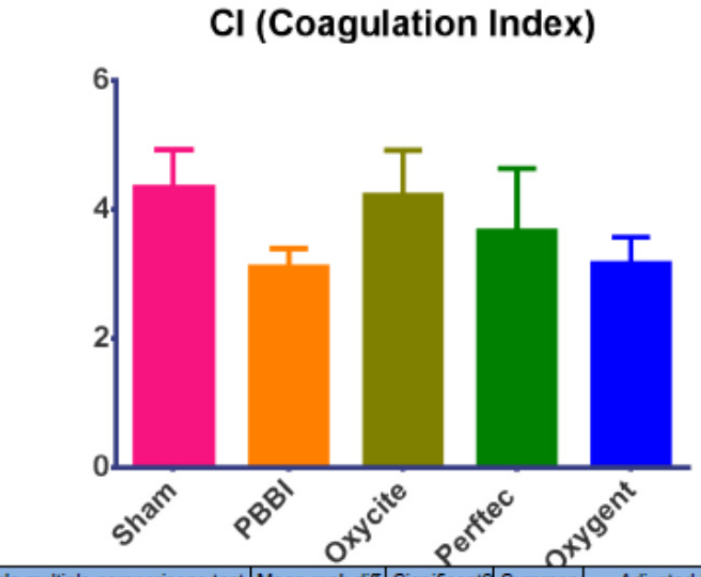
Fig 14. TEG values for K (Potentiation phase of enzymatic factors yielding clot strengthening via thrombin cleavage of fibrinogen into fibrin) not significantly different with PFCs onboard after PTBI.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
Sham vs. PBBI	-5.083	No	ns	> 0.9999
Sham vs. Oxycite	-3.833	No	ns	> 0.9999
Sham vs. Perftec	-6.900	No	ns	0.7610
Sham vs. Oxygent	-7.357	No	ns	0.5042



Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
Sham vs. PBBI	8.667	No	ns	0.3529
Sham vs. Oxycite	0.5000	No	ns	> 0.9999
Sham vs. Perftec	2.533	No	ns	> 0.9999
Sham vs. Oxygent	2.476	No	ns	> 0.9999

Fig.15 TEG values for the G (overall clot strength resulting from all coagulation interactions, calculated from amplitude (A), G=(5000xA)/(100xA), the process of clot dissolution or fibrinolysis, leads to a decrease in (G) not significantly different with PFCs onboard after TBI.



Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
Sham vs. PBBI	7.000	No	ns	0.6611
Sham vs. Oxycite	3.083	No	ns	> 0.9999
Sham vs. Perftec	3.600	No	ns	> 0.9999
Sham vs. Oxygent	4.286	No	ns	> 0.9999

Fig.16 All TEG values CI (CI is linear combination of R,K,MA and α. CI=-0.1227R+0.0092K+0.16655MA-0.0241α−5.0220) not significantly different with PFCs onboard after PTBI.

Model	PBBI	FPI	ASDH
Human equivalent	Gunshot	DAI and small contusion	ASDH with large ischemia
TEG data on 2.5h after injury (Compared to Control)	R↑ K↑ α↓ MA↓G↓CI↓	No severe change	R↑↑ K↑↑ α↓↓ MA↓↓ G↓↓ CI↓↓
Peak / recovery of coagulopathy	-	Peaked on 24h Recovered by 7d	Peaked on 2.5h post injury Recovered by 7d
Hypotension / shock	None		
Hemoglobin (2.5h)	No significant difference		
Platelet counting (24h)	No Significant difference		
Neurodegeneration measured with FJB (2.5h- <24h post injury)	++	+	+++
Translation	Early (2.5h), mild Enzymatic coagulopathy, Fibrin dysgenesis Platelet–fibrin dysfunction,	Late (24h) Enzymatic coagulopathy, Fibrin dysgenesis	Early (2.5h), severe Enzymatic coagulation, Fibrin dysenesis Platelet–fibrin dysfunction,
Possible primary cause of coagulation disorder	Moderate tissue injury, Acute tissue factor (TF) release?→ Moderate Coagulopathy	Late onset secondary axonotomy?→ Late onset coagulopathy	Severe tissue injury → Higher TF release? → Severe consumptive coagulopathy

Table 1. Data summary and possible pathomechanisms.

PROGRESS WITH AIM 3.

1. Changes in VO_2 in FPI + secondary hypoxia model were different from that of PBBI. No significant differences existed between right and left hemispheres of uninjured animals. The VO_2 levels were depressed in vehicle treated injured group in FPI + secondary hypoxia as seen in PBBI compared to uninjured group. Perftec administration improved the VO_2 both on the ipsi and contralateral sides. However, it did not differ significantly from either the uninjured or injured. The counting of the Fluoro jade positive cells for the Aim 2 (see Fig.3B) shows that the improvement in VO_2 is not sufficient to translate into cell survival in that model unlike with TBI alone. In summary, the PFCs were associated with slightly improved oxidative brain metabolism, but there was no statistically significant difference between the PFC's in PBBI or FPI+secondary hypoxia. (PFC1=Oxycyte PFC 2 = Perftec)

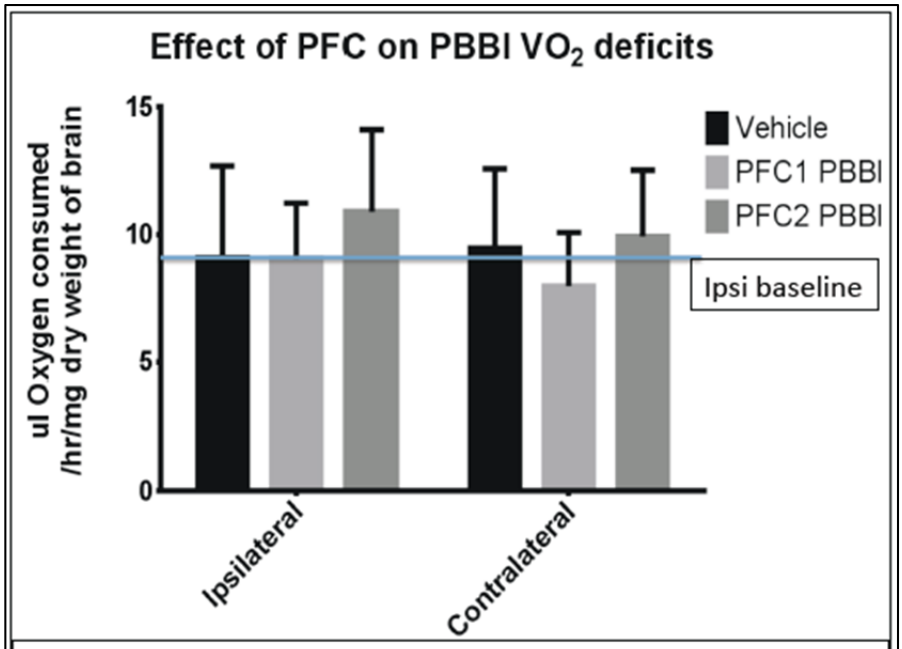


Fig. 17 No effect of PFC1 on the VO_2. The x-axis shows the brain hemisphere with respect to injury, the y-axis shows the units of O_2 consumed (VO_2). The VO_2 in animals treated with vehicle (black bars) were not statistically significant from those treated with Oxycyte (light gray bars) and Perftec (dark gray bars) on the ipsi or contralateral sides.

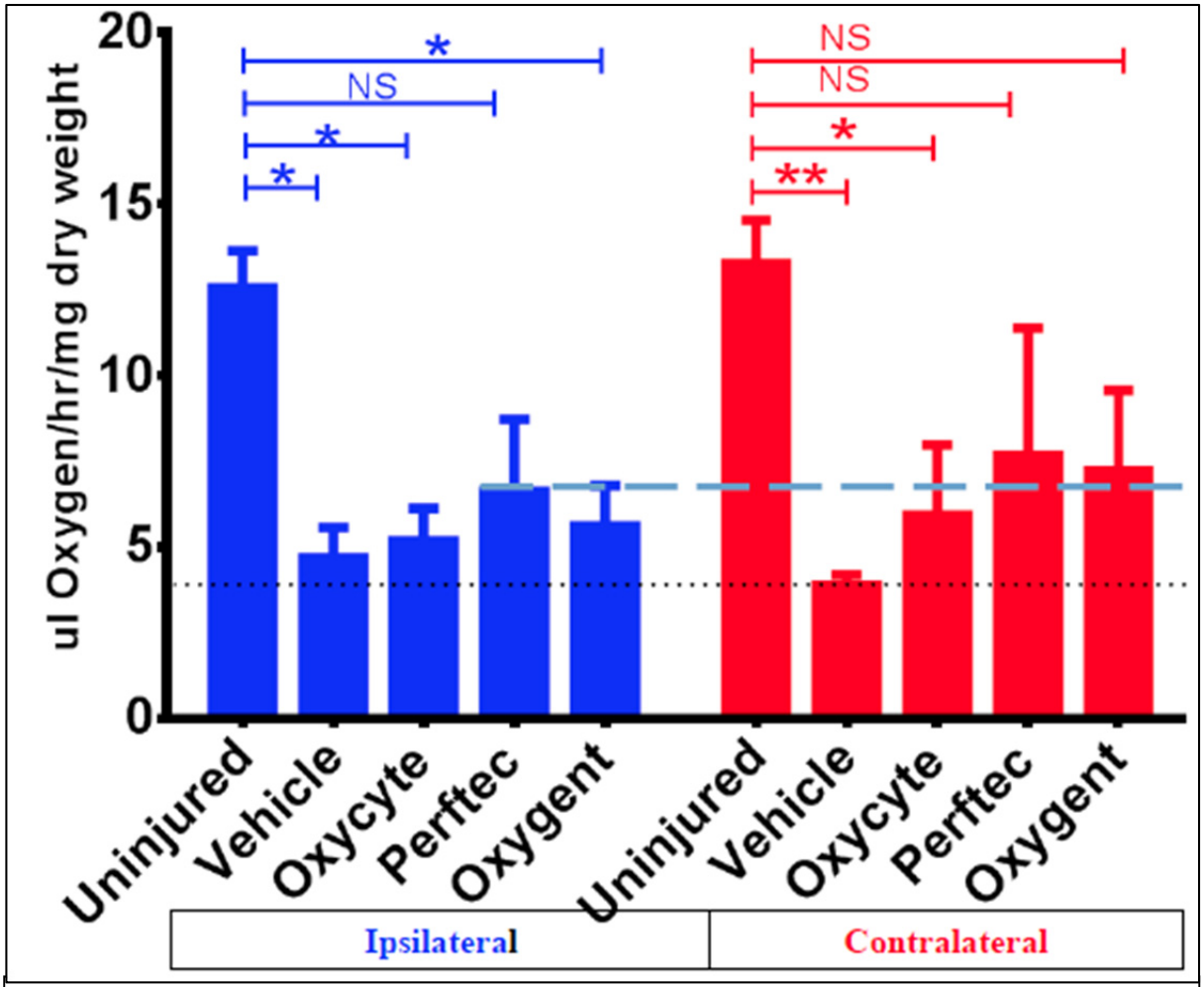


Fig.18 Perftec ameliorates VO_2 after TBI+secondary hypoxia. Ipsilateral to injury VO_2 in Perftec treated animal cortices is not significantly different than sham. Dunnett's multiple comparison test *=p<0.005. The improvement is greater on the contralateral side.

2. PTBI significantly reduced both oxygen consumption, and glucose use (based on ^{14}C 2-deoxy glucose), in the hemisphere of the lesion (ipsi). This study documents, for the first time, the severity and spatial distribution, of these changes in the brain after PTBI, in this rat model (Fig 17,19-22). Equipped with data on PBBI global ischemia and neurodegeneration data, we asked of how the spread of global ischemia after PBBI at 2.5h translates into neurodegeneration at 24-72h? The assessment presented in Fig 19-20 shows that the spread of PBBI induced global ischemia is far greater than the neurodegeneration (almost twice). In the 4mm region along the rostro-caudal axis, there is a less than 2-fold difference in percentage of glucose depression between the core (-0.3mm from Bregma) and peri-lesional area (-4.3mm from Bregma) while in contrast there is a 10-fold drop in FJB-positive cells. Thus in this 2mm region -0.3mm to -2.3mm ischemia directly translation into cell death. However, in the next 2 mm (-2.3 to -4.3mm Bregma) there is a dramatic decrease in cell death (34% at -0.3mm Bregma to 3.4% at -4.3mm Bregma) despite almost similar ischemia (34% vs. 20%). Taken together the data suggest that even in PTBI there is a window of opportunity for therapeutic intervention (2.5-72h) and not all tissue subjected to PTBI is destined to perish. However with PFCs did not significantly increase tissue sparing in this study.

Group	Sham	Minimal injury	Severe injury	Severe injury	Severe injury
Probe inflated	None	No	Yes (10%)	Yes (10%)	Yes (10%)
Treatment	None	None	Vehicle	Oxycyte	Perftec

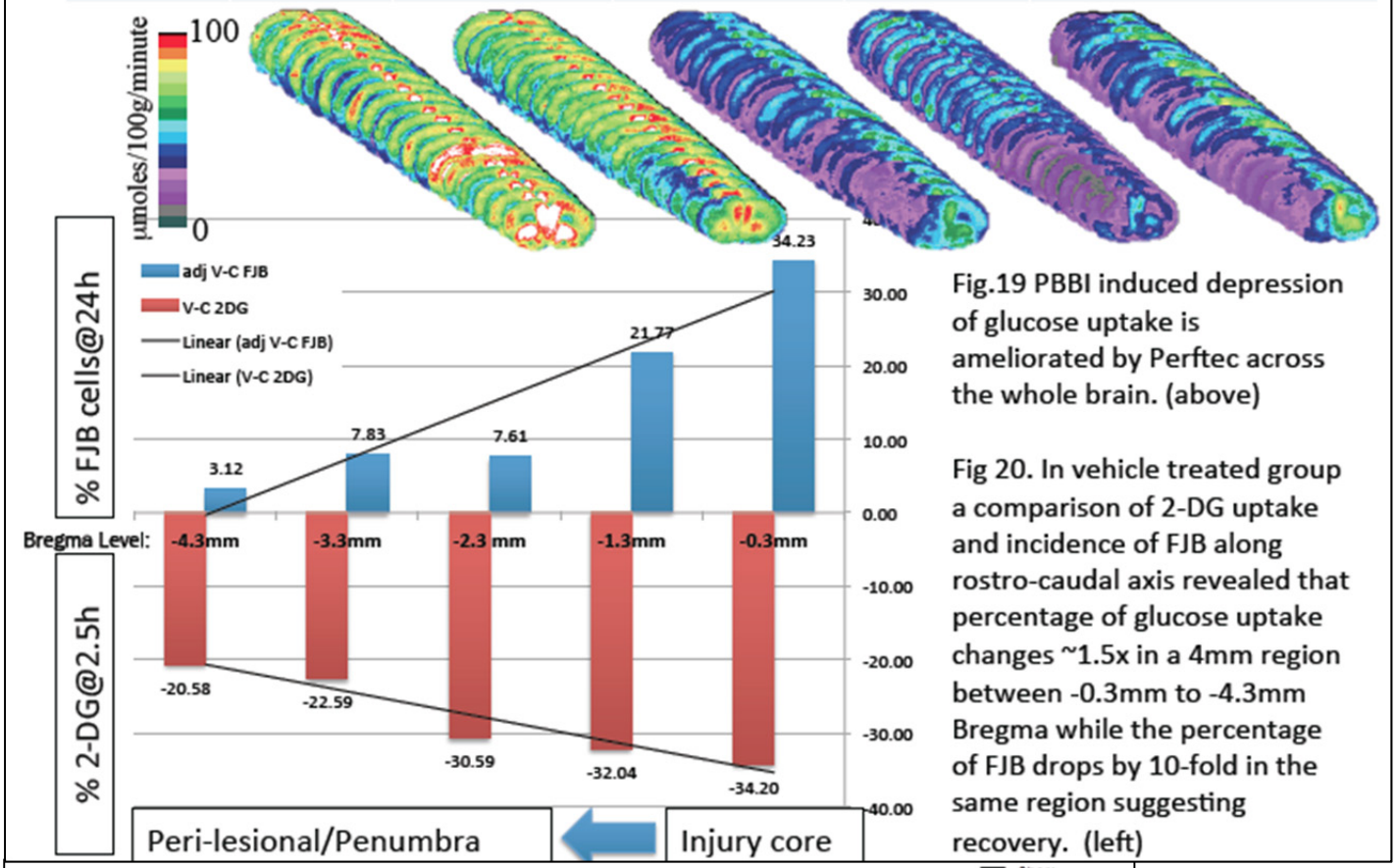


Fig.19 PBBI induced depression of glucose uptake is ameliorated by Perftec across the whole brain. (above)

Fig 20. In vehicle treated group a comparison of 2-DG uptake and incidence of FJB along rostro-caudal axis revealed that percentage of glucose uptake changes ~1.5x in a 4mm region between -0.3mm to -4.3mm Bregma while the percentage of FJB drops by 10-fold in the same region suggesting recovery. (left)

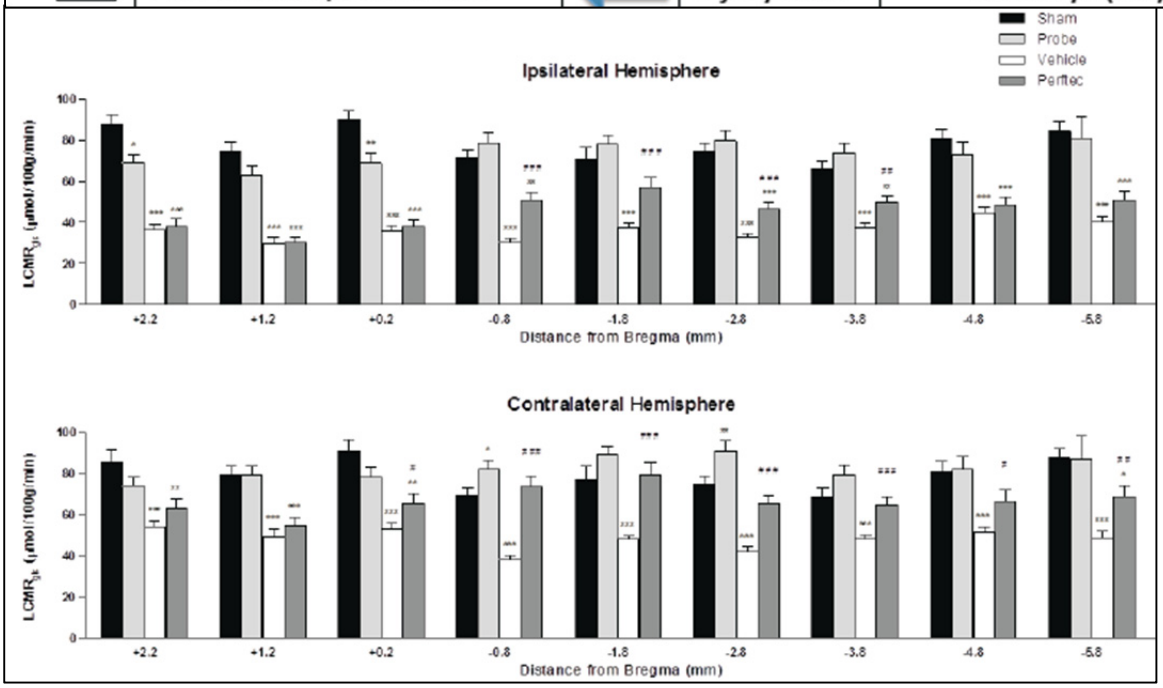


Fig.21. Glucose utilization (radioactive 2-deoxy glucose signal) is reduced 2.5h post PBBI but significantly restored by Perftec. The effect is seen both ipsi- and contra-lateral to the injury. The recovery of glucose uptake is posterior to injury at - 0.8mm Bregma.

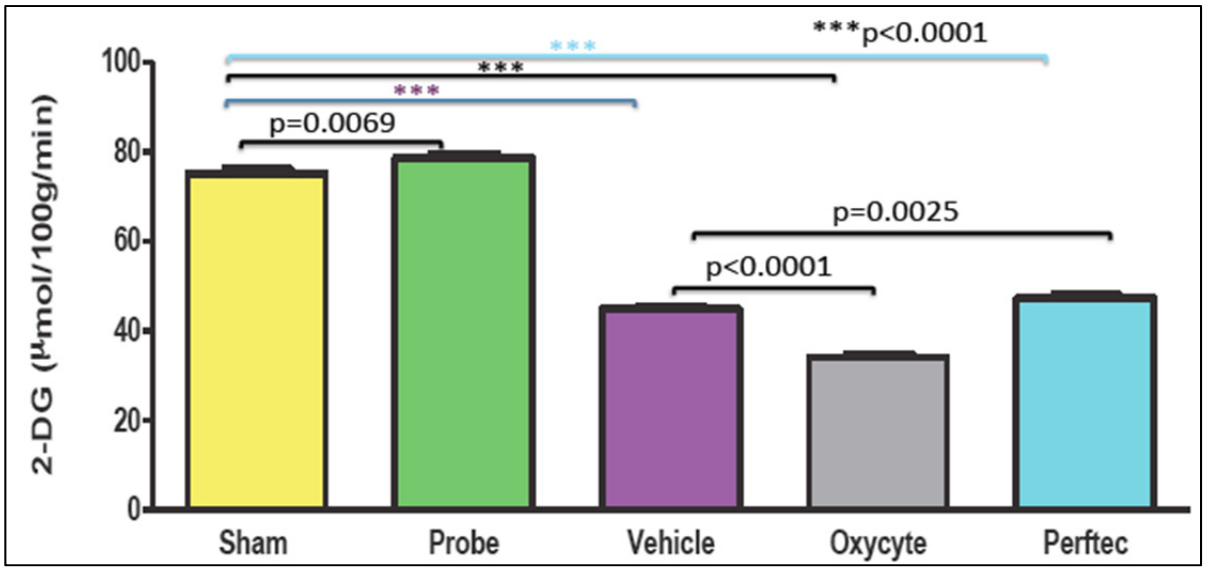


Fig.22 Quantitative analysis of 2-DG in PBBI bra8in treated with PFCs. PBBI induces significant depression of 2-DG uptake (Sham vs Vehicle), not seen with uninflated probe only (Sham vs Probe). Perftec ameliorates depression of 2-DG uptake significantly (Vehicle vs Peftec). The increase is however still significantly different from Sham.

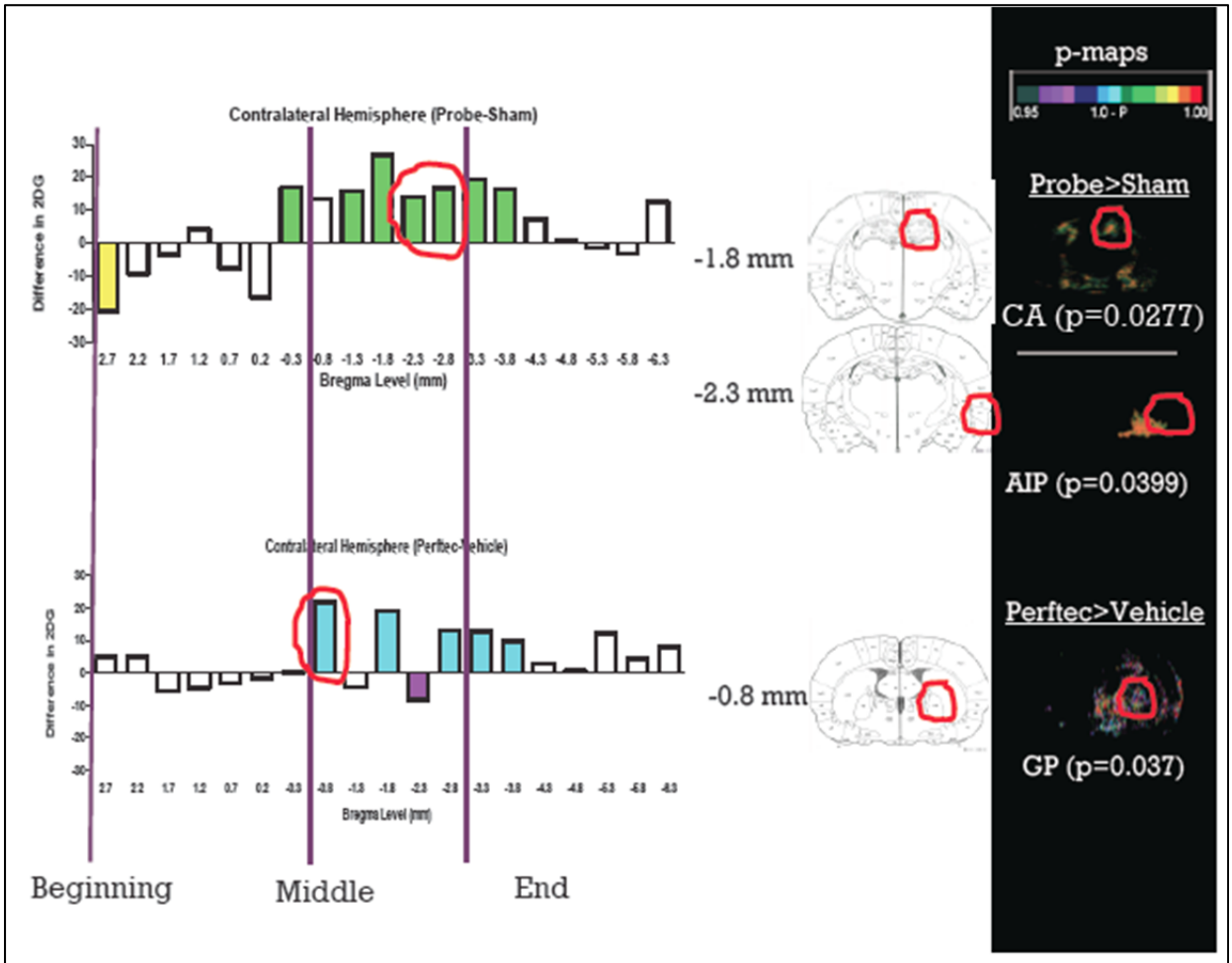


Figure 23. Pixel based "p mapping" method, shows that Perftec amelioration of glucose use is seen near the lesion.

Effect of 3 different PFC upon Glucose use, after both Penetrating TBI, and Fluid percussion injury. (Aim 3B) Significant and robust improvements in glucose use were seen in multiple brain regions, associated with Perftec administration, when compared to vehicle treated animals (Fig.19-23). Figure 23. Pixel based "p mapping" method, shows that Perftec amelioration of glucose use is seen near the lesion. The effect of closed head trauma upon Glycolysis, as measured by the 2-Deoxyglucose method, is well known, and the findings in this model accord quite closely with human TBI. However, the effect of Penetrating TBI upon glycolysis has never been studied, in any animal model, nor in humans. We have robust findings, concerning the effect of PTBI upon glucose use, which will be the focus of a future paper. In brief, PTBI was associated with profound reductions in glucose use, both globally, in the whole brain, and focally around the PTBI site, at 2 hours after injury. Surprisingly, no significant hyperglycolysis was seen, in contrast to other animal models.

Unfortunately, no robust, ameliorative effect of any of the 3 PFC's tested was seen upon VO_2 in the PBBI model (Fig.17-18) However, significant improvements in glycolysis could be observed, especially with Perftec and Oxycyte after PBBI (Fig. 19-23). **After PTBI, administration of 2 different PFC's was associated with significant amelioration of this depressed glycolysis (Fig 19-23)**

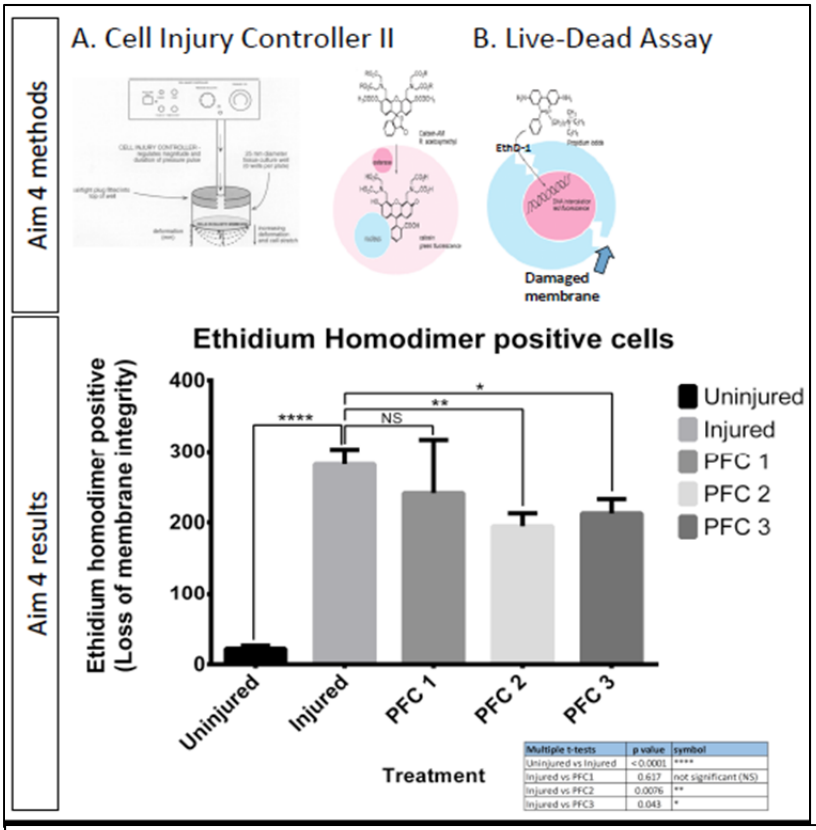


Fig. 24. A summary of the methods (top), experimental set-up of the Cell Injury Controller II is shown in A. The schematic on right shows the principle of live-dead assay in B. Calcein-AM enters intact cells and is retained by a viable cell and appears fluorescent green color under a microscope. Membrane impermeant Ethidium homodimer (EthD-1) enters the cells through the damaged membrane (e.g., due to loss of membrane integrity) and appears red fluorescent after binding to cellular DNA. Confocal imaging and counting of $EthD1^+$ cells was employed. Analysis of results is represented by the graph (left).

This is a counter intuitive, but important finding, which deserves further study, and may open a new mechanism, by which PFC may improve recovery, after PTBI in particular, and TBI in general. This finding was seen in tissues distant from the injury epicenter, but in both hemispheres, after PTBI. This suggests that the rate limiting enzymes for the glycolytic pathway, (chiefly hexokinase) are influenced by PFC, either directly, or via an increase in oxygen tension, in the tissue. With regards to the global glucose depression in a recent insight we found that activators of innate immunity such as Caspase 1 specifically target glycolytic enzymes [6], the

glycolysis data is consistent with global induction of Caspase 1 following PBBI at the earliest time point analyzed i.e., as 4h and the peak is reached around 48h. These results will be discussed at NNS and MHSRS 2014.

AIM 4. MILD TBI.

We have a five replicate experiment completed; with each PFC and saline controls, after stretch injury, imaging and counts are ongoing, for that experiment. We have standardized the imaging of the stretch injured cells while still on the thick silastic membrane. This is the first time such experiments have been done to our knowledge. The quantitation of the data revealed that the **Perftec and Oxygent were associated with significantly reduced cell death in these cultures, 24h post stretch injury.** The effect of 2 of the 3 PFC's studied here seems to be independent from its ability to dissolve gases, since these cells were growing

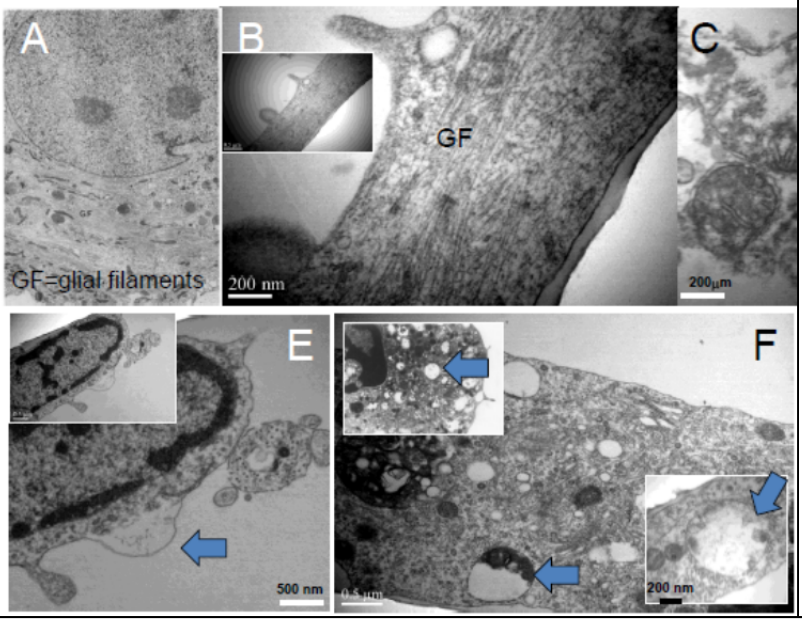


Fig. 25. Electron microscopic examination of stretch injury cultures at 24h post PFC treatment show disruption of glial filaments (GF) in (B) consistent with those published by *Ellis et al.,* 1995 (A). In severely injured cells disrupted mitochondria can also be seen (C). PFC appears to fuse with cell membrane or pinocytosed (arrow in E), inset shows low mag of the same image (E). PFCs within cytoplasm appear to be sequestered in vesicles (arrows in F, lower right inset shows PFC vesicle at higher magnification) similar to that seen with fluosol, a different PFC (upper left inset) reported by *Ingram et al.,* 1992. Magnification is indicated by the micron bars.

in a fully oxygenated supernatant growth medium solution. Numerous membrane-sealing agents are being developed for use in acute TBI; ability to functionalize such molecules with PFCs may increase their utility--more experiments are warranted. [7, 8]

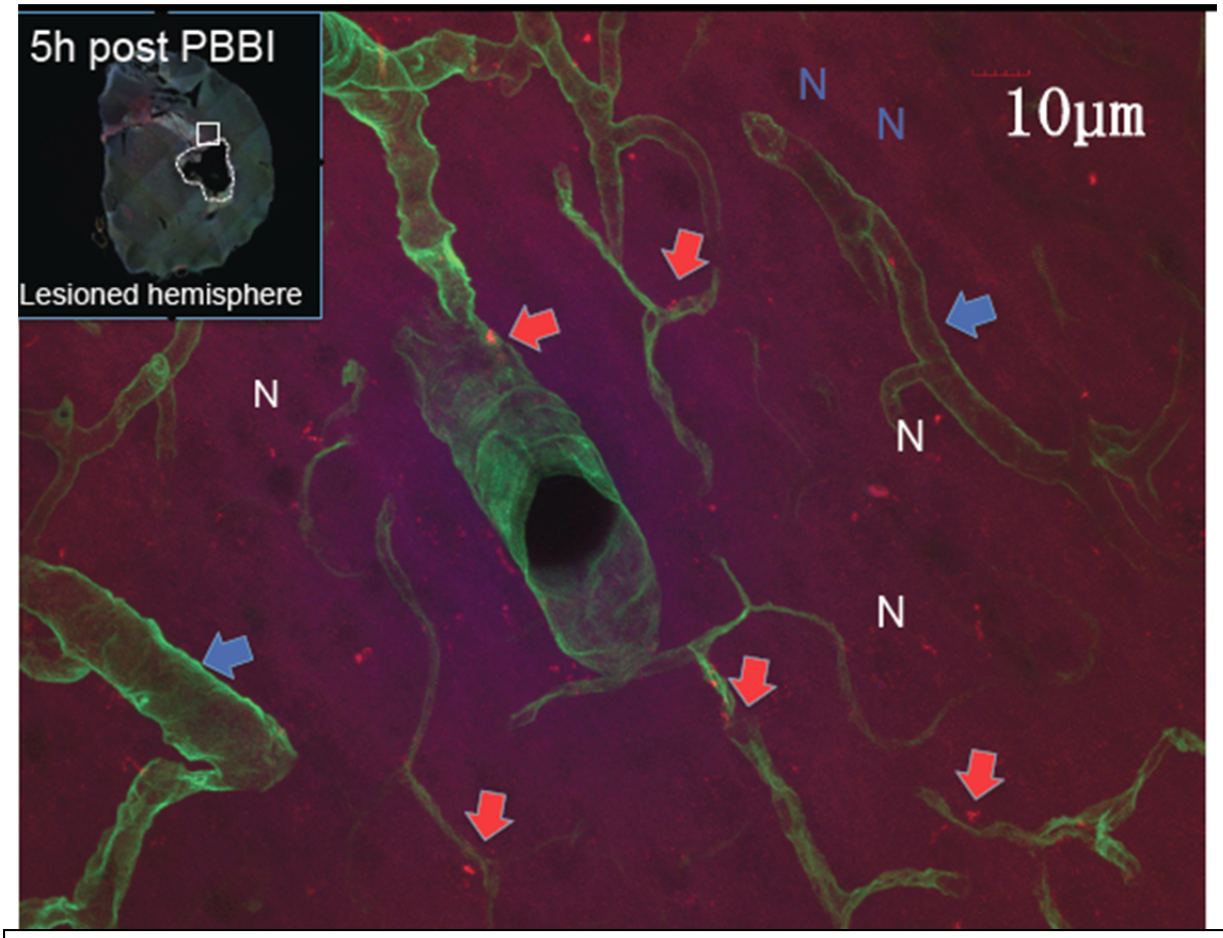


Fig.26. Ethidium homodimer labeling of membrane permeablizied cells in vivo after PTBI. The EtHD labled squamous epithelia of vasculature (green) is pointed by red arrows, such vasculature is collapsed or narrow. Health vasculature (green) without loss of membrane integrity is pointed out by blue arrows. Similarly white N shows membrane permeabilized neurons while a blue N shows intact neurons. The inset shows a low magnification image of the entire lesioned hemisphere (dotted outline) and the white square corresponds to the area shown.

To further, assess the utility of the live dead assay to uncover the loss of membrane integrity following PBBI (5h, a time point that has most mature primary injury, just before infiltration of the neutrophils into the lesion) we applied ethidium homodimer to an injured animal and perfused it with Dylight 488 labeled lectin. Serial sections showed the presence of ethidium homodimer in the nucleus of squamous epithelium of the blood vessels, more often than not such vessels were either completely ruptured or collapsed, similarly ethidium homodimer was observed in some parenchymal cells too. The labeling was absent in healthy tissue and in intact blood vessels (see Fig 26). Taken together these data suggest that PBBI mimics the primary

injury in PTBI, i.e., loss of membrane integrity, however the benefit seen with PFC *in vitro* could not be replicated *in vivo,* suggesting that the immune system or glia may be exacerbating the primary injury *in vivo*.

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(3) SECTION IV - A DESCRIPTION OF WORK TO BE PERFORMED AS A CONCLUSION OF THE FUNDING PERIOD.

Manuscripts to be prepared, and supported by putatively publishable data

1. Effect of PTBI upon Cell death patterns. Fluoro jade staining via unbiased stereology data- never reported.
2. Relationship between TEG and severity of brain damage in 3 different animal models, of TBI
3. PFC ameliorates anaerobic glycolysis, but not oxygen metabolism, after PTBI, and closed TBI, in rat models.
4. Histopathological correlates of spatial and anatomical patterns of alteration of glycolysis, after PTBI.

Respectfully Submitted,
R Bullock/Shyam Gajavelli
March 31 2014.

The Role of Perfluorocarbons in Mitigating Traumatic Brain Injury.

Insert DMRDP Proposal Number, Project and Task title here

PI: M. Ross Bullock, MD, PhD **Org:** Miami Project to Cure Paralysis, University of Miami **Award Amount:** $$$$$$$

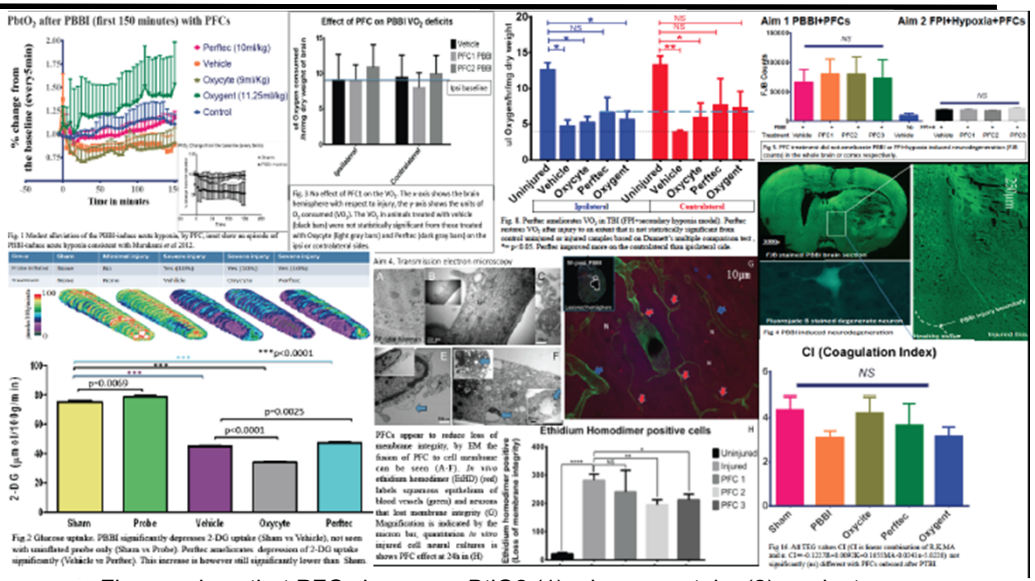
DMRDP

Study/Product Aim(s)

1) PFC will be effective in mitigating PPBI, as tested in the WRAIR/Tortella model, with acute brain histology, at 24 and 72 hours after injury, in the rat.
2) Two doses of PFC, given 24 hours apart will be safe and effective in mitigating secondary ischemic damage, superimposed upon severe closed TBI in the rat. Thrombolesatography (TEG) to uncover PFC adverse effects on coagulation.
3) PFC's will improve both oxygen consumption ($CMRO_2$) and glucose uptake, in the rat brain, after PTBI.
4)PFC will improve cell survival, in an *in vitro* model of MILD TBI, when applied in the supernatant culture medium.

Approach

Animals were subjected to PBBI or TBI+hypoxia randomly assigned to groups treated with (1) vehicle, (2) PFC1, (3) PFC2, (4) PFC3. Elevation of O_2 following PFC administration assayed using Licox probe. Glucose uptake assessed with radioactive 2-deoxy glucose, to assess oxygen consumption brain cores were assayed by microrespirometry, Fluorojade B staining of brain sections to uncover neurodegeneration and stereology to quantitate cell death. Live-dead assay of postnatal cultures subjected to *in vitro* TBI in the presence of PFC to assess loss of membrane integrity.



Figures show that PFCs improve pBtiO2 (1), glucose uptake (2),modest improvements in VO2 (3), some membrane preservation (4) but these effects do not translate to mitigation of neurodegeneration (5) or traumatic coagulopathy (6).

Timeline and Cost

Activities FY	11	12	13	14
1) Neurodegeneration in PBBI brains with Fluorojade B stains		Animals; Work Product		
2) PFC mitigation of secondary damage (TBI+hypoxia) Thromboelastography		Animals; Animals; Work Product; Work Product		
3) Glucose uptake and VO_2 following PBBI.		Animals; Work Product		
4) PFC mediated mitigation of membrane integrity loss		Animals; Work Product		
Estimated Budget ($K)	**$0 00**	**$587,752**	**$557,490**	**$232,582**

Updated: 03/31/2014

Goals/Milestones

FY11 Goal – IACUC ACURO approvals, transfer PBBI machine, train
☑ PBBI model established at UM, trained postdoc and students.
FY12 Goals – Animal surgeries for Aim 1 , 3
☑ Completed Aim surgeries, neurodegeneration studies ongoing.
☑ Completed radioactive glucose uptake surgery.
☑ Set up Aim 3 microrespirometry animal and experiments.
FY13 Goal – Aim 2 microrespirometry, Aim 4 experiments
☑ Aim 2 animal surgeries and microrespirometry completed.
☑ Aim 2 histology completed
☑ Aim 4 imaging and data collection completed.
FY14 Goals – Communication of results
☑ Abstracts at NNS, MHSRS
☑ Publish manuscripts in peer reviewed journals.
Comments/Challenges/Issues/Concerns
- None.

Budget Expenditure to date

Projected Expenditure: $1,976,684 Actual Expenditure: $1,922,212